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Effect of *Bacillus thuringiensis var krustaki* on the mortality and development of *Culex pipiens* (Diptera; Cullicidae)

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Abstract
The bacterium *Bacillus thuringiensis*, is one of the insecticides mostly used at the moment as agent of biological control to suppressing the populations of diverse insect pests. The present study tested the strain *Bacillus thuringiensis var krustaki* against fourth stage larvae of *Culex pipiens* where they were exposed to different concentrations of *Btk* (25mg/l, 50mg/l et 83mg/l) for 48h, 120h, 240h and 360 hours. High larval mortality was recorded; this toxicity is expressed in terms of dose and time. Lethal concentrations LC_{50} calculated after 48 hours is 380.19 mg/l and the LC_{90} was 8912.51 mg/l. However, at 360 hours of the treatment, the CL50 % and the CL90 % does not exceed the 52.48 mg/l and 107.15 mg/l.
Btk disrupts fecundity and fertility of adults issued from treated larvae, with an average low eggs of 33.20 ± 5.116 induced by the dose of 25 mg/l and low hatching duration of eggs with 2.30 ± 0.48 %.

Keywords: *Btk*, *Culex pipiens*, toxicity, fertility, fecundity

1. Introduction
Of all disease-transmitting insects, the mosquito is the greatest menace. Spreading malaria, dengue, and yellow fever, all together is responsible for several million deaths and hundred million cases every year [1]. Over the last 50 years, insect pests control has mainly been with synthetic chemical insecticides such as organochlorines, organophosphates, carbamates, and pyrethoids, of which organophosphates and carbamates are the major classes in use today [2-3]. However, problems due to pesticides resistance, negative effect on non-target organisms (including humans and the environment) [4], led to the research for new solutions to fight against these insects pests biologically.
Biological control is the use of living beings (control auxiliaries) or their products (inert biopesticides) to fight pests or disease vectors. While inert biopesticides are generally derived from bacteria or fungi and they are the quickest biodegradable [5], Bacillus thuringiensis variété krustaki (Btk) is one of these inert biopesticides. It is a bacterium recognized by its protein crystal included in the cytoplasm (O-endotoxin) which is toxic to mosquito larvae [6]. This crystal which constitutes the larvicidal product is presented in the form of a wettable powder or a concentrated solution. The larvicidal action is therefore due to a chemical substance of biological origin produced by this bacterium. Bacillus thuringiensis israelensis (Bti) has demonstrated a significant larviciding effect on more than 115 species of mosquitoes and 40 species of black flies [7].
In this study, we are interested in both direct and indirect effects of this product on the most abundant species of Culicidae in Algeria: Culex pipiens. The present study aimed to investigate the impacts of the insecticide on mortality and development of Cx. pipiens.

2. Material and Methods
2.1. Insects
Cx. pipiens is a cosmopolitan species that acts as a major vector in the transmission of many diseases. It is considered as an important nuisance to human populations exposed to its bites in most African countries [8]. Its development cycle lasts 10 to 14 days and includes four stages: egg, larva, pupa, and adult. The initial stages (egg, larva and pupa) are adapted to the aquatic lifestyle while the final stage is aerial [9].

2.2. Mosquito Rearing

The larva used in this study provokes a mass livestock of adults collected in urban areas of Annaba, Eastern Algerian city. Livestock is kept in laboratory cages (20 x 20 x 20 cm) at a temperature of 25 ± 2 °C, humidity of 75 ± 10 % and a 12-hour scotophases. A mixture of biscuit and dried yeast insures the nutrition of larvae, while the adults feed on dried raisins.

2.3. Bacillus thuringiensis var kurstaki (Btk*)*

Is a positive Gram bacterium which has the feature of synthesizing a protein crystal, of a cubic or bipyramidal structure, during sporulation? The crystals have larvicidal activity on different species of insects. The poisoning manifests itself very rapidly by major lesions in the intestine and by a paralysis of the digestive tract [10]. For this study, we used a commercial product, in the form of a powder containing 23,000 IU.

2.4. Treatment

Treatment of *Culex pipiens* was inspired by the technique of standardized sensitivity tests of the World Health Organization [11].The tests are carried out in beakers with a capacity of 200 ml each containing 10 fourth-stage larvae of *Cx. pipiens* (L4) in 200 ml of spring water. After a preliminary test, three concentrations of the L4 stage larvae (23 mg/l, 50 mg/l, and 83 mg/l) were administered. These values are chosen in order to get the lethal doses 100 (LD100). Each concentration is applied to 3 repetitions, with a preparation of 10 larvae of *Cx. pipiens* as a control.
After 24 hours, the water of treated groups as well as of the control one has been changed. We daily note the number of the dead individuals (L4 larvae, nymphs and adults).

2.5. Effects of *Btk* on fecundity and female fertility

Adults that have completed their development and that are from larvae treated with the lowest dose (23 mg/l), were isolated and separated in couples (male and female) in cages (20 x 20 x 20 cm) which include water containers. After coupling, the number of laid and hatched eggs for each female is counted. The results were subject to statistical description and comparison of variances.

2.6. Data Analysis

For lethal concentrations and lethal times (LC_{50} % LC_{90} %, LT 50% and LT 90%) were calculated using the Finney's [12] mathematical method. Data are normalized and processed according to the tables of Bliss, and calculations were performed on XLStat 2009 Software.
Also, the results of fecundity and female fertility were subject to statistical description and comparison of variances using XLSTAT2009 Software.

3. Results

3.1. Effects of *Btk* on the mortality of *Cx. pipiens*

The obtained results showed that the *Btk* affects the mortality of larvae based on the applied concentration and exposure time. Low larvicidal activity was recorded for the concentration of 23 mg/l. regarding the concentration of 83mg/l, more than 80% of treated individuals die after 15 days of treatment, and there are no significant differences between the recorded mortality rate and the used concentrations (Table1).

Table 1: Mortality rate (%) of *C. pipiens* treated with *Btk*

Concentration (mg/l)	2 Days	5 Days	10 Days	15 Days	F_{obs}	*p*
23mg/l	0	6.67	10	13.33	4.405	0.04*
50mg/l	10	13.33	23.33	30	2.584	0.126
83mg/l	33.33	73.33	83.33	86.67	1.274	0.347
F_{obs}	16.00	2.255	1.532	1.390		
p	0.004*	0.186	0.290	0.319		

(*: Significantly different)

Larvae mortality rates are positively correlated with the used concentrations of *Btk* (R^2 = 0.80 to 1.00) (Table 2A). Two days after treatment, the lethal concentration of 50% was 380.19 mg/l and it decreased to 69.18 mg/l in 5 days, 57.54 mg/l in 10 days and 52.48 mg/l in day 15. The mortality of 90% of the larvae may be caused with concentrations ranging between 107.15 mg/l and 8912.51 mg/l (Table 2A).
There is also a strong correlation between mortality and the larvae's exposure time to different concentrations of *Btk* (R^2 = 0.001 to 0.94) (Table 2 B). Calculated lethal times range from 72 to 1738 days for a 50% mortality (Table 2 B) and vary between 389 and 51286 days for LT 90% (Table 2 B).

Table 2: Toxicological parameters of *Btk*; A: Based on larvae's exposure time / B: Based on the used concentration

A				
Time (days)	**2**	**5**	**10**	**15**
Regression line	Y=2.57+0.94X R²= 1.00	Y= -2.21+3.91X R²= 0.82	Y= -2.34+4.18X R²= 0.86	Y=-2.09+4.13X R²=0.88
LC_{50}% (mg/l)	380.19 mg/l	69.18 mg/l	57.54 mg/l	52.48 mg/l
LC_{90}% (mg/l)	8912.51 mg/l	147.91mg/l	114.81 mg/l	107.15 mg/l

B			
Concentration (mg/l)	**25**	**50**	**83**
Regression line	Y=-6.66+4.34X R²=0.81	Y=2.18+0.87X R²=0.94	Y=5.14-8.04X R²=0.001
LT50% (j)	1738 d	490 d	72 d
LT90% (j)	51286 d	955 d	389 d

y: probits of mortality rates; x: the logarithm of the concentration and/or time

3.2. Effect on fecundity and fertility

After treating the fourth stage larvae by sub-lethal concentration of 25 mg/l, the present study noticed a longer larval life, a disturbance of the fecundity and fertility of adults from this treatment.

3.3. Effect on the number of eggs

The results in the table (Table 3) show that the females treated with *Btk* lay on average 33.20 ± 5.116 eggs with a minimum of 24 eggs and a maximum of 42 eggs; however. Healthy females lay between 33 and 59 eggs. Comparison of eggs means showed that there were no significant differences between the fertility of the two lots (t = 2.71; *p*: 0.153) (Table 3).

Table 3: Comparison of eggs from control females of *Cx. pipiens* and eggs from females treated with *Btk* (n = 10).

	Mean ± S	Min	Max	t_{obs}	*p*
A	46.80 ± 8.43	33	59	**2.71**	**0.153**
B	33.20 ± 5.116	24	42		

A: Number of eggs from the control females; B: Number of eggs from females treated with Btk. Mean ± s: mean ± standard deviation; Min: Minimum; Max: Maximum; *: Significantly different

3.3. Effect of *Btk* on the hatch

Control females take longer time to hatch the eggs; their average time is 2.9 ± 0.57 days with a maximum duration of 4 days and a minimum of 2 days (Table 4). Females treated with *Btk* take less time (2.30 ± 0.48days) to lay the eggs with duration of three days (Table 4). It has been recorded also that the hatching rate among control females (90.37%) is higher than that of the treated females (68.85%) (Table4).

Table 4: Effects of *Btk* on the eggs hatch of *Cx. pipiens* females.

	Hatch duration			Hatch rate (%)		
	Mean ± s	Min	Max	Mean	Min	Max
A	2.9 ± 0.57	2	4	90.37	80	98.21
B	2.30 ± 0.48	2	3	68.85	34.28	91.42

A: The eggs of the control females; B: The eggs of females from larvae treated with Btk. Mean ± s: mean ± standard deviation; Min: Minimum; Max: Maximum

4. Discussion

Bti is considered the reference larvicidal for mosquito control operations at lethal doses; no resistance mechanism has been reported. These toxins remain harmless to other insects, fish and animals [13]. To note that this day, no mechanism of resistance was detected at mosquitoes, essential toxin of the crystal acting in synergy [14].

The present study conducted a toxicological study by using *Btk* on the larvae L4 which showed a good larvicide activity on *Cx. pipiens*, translated by rates of high mortality. The insecticidal activity of *Btk* is progressive because of increase in the mortality as we move forward in the time of exhibition to achieve for 90 % mortality for the highest concentrations.

Several works as those of [15-16] have already demonstrated larvicide effect of *Btk* on *Cx pipiens, Cx univittatus Ae aegypti, Ae rusticus* and *Anopheles.* Other works indicates the effect of this bioinsecticide on several groups of insects (Diptera, Dictyoptera, etc.) such as the study of *Habbachi and al*., (2014) [17] who reveal the consequence of the *Btk* concentrations on the development of *Drosophila melanogaster* and those of Martouret & Auer, 1977 and Lam & Webster, 1972 [18-19] to the *Zeiraphera diniana* and the European crane fly.

The current study also recorded the indirect effects of *Btk* on *Cx. pipiens*. The treatment with the low concentrations (23 mg /l) caused a disruption in the fertility and fecundity at the adults stemming from this treatment.

The reduction of the fertility and the rate of hatching to the handled experimented females would be under the influence of properties of *Btk*, *Btk* is a protein crystallized during the stage of sporulation of its life cycle. The process of toxicity is especially important by ingestion; in topical application, *Btk* presents a brief half-life because it is quickly inactivated [20]. To the larvae of Lepidoptera, These microscopic crystals are ingested and transformed into toxic protein molecules (of-endotoxin) which destroy stomach walls; insects usually stop feeding within hours which follow their exhibition (exposure) in *Btk* and die usually 2 in 5 days later [21].

5. Conclusion

In the laboratory, we have demonstrated the toxic effect of *Btk* on *Cx. pipiens* larvae. The mortality observed was positively correlated with *Btk* concentrations and duration of exposure. This study also shows that *Btk* ingested by larvae causes disturbances in the fecundity and females fertility. This subgates direct effects of the treatment on larvae survival and indirectly on the disturbance of reproduction process. All the potentialities of this bacterium are not still known probably not and numerous researchers, in the world, strive or to discover new activities against different species of mosquito or still to improve the already used origins. Therefore, *B. thuringiensis* presents real perspectives of development.

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